Ecotoxicology and Environmental Safety 219 (2021) 112312

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Elucidating the effects of TiO_2 nanoparticles on the toxicity and accumulation of Cu in soybean plants (*Glycine max* L.)

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ARTICLE INFO

Edited by Richard Handy

Keywords:
TiO_2 nanoparticles
Cu
Phytotoxicity
Accumulation
Adsorption

ABSTRACT

Copper (Cu) pollution is common in the soil. Due to the widespread application of TiO_2 NPs, there is a high propensity for the co-occurrence of TiO_2 nanoparticles (NPs) and Cu in agricultural soils. It is therefore imperative to evaluate the joint effects of TiO_2 NPs and Cu on crops. In this study, the mutual effects of TiO_2 NPs and Cu on their toxicity and accumulation in soybean seedlings and on their fates in a hydroponic system were determined. When Cu was at levels of 1 and 2 mg/L, the co-occurring TiO_2 NPs at a non-toxic concentration (10 mg/L) significantly enhanced the toxicity and accumulation of Cu and Ti in soybeans, and inhibited the translocation of Cu from soybean roots to shoots. However, when the Cu concentration for co-exposure was $\geq$ 5 mg/L, such mutual effects disappeared. The amount of Cu ions adsorbed onto TiO_2 NPs after 48 h of co-exposure gradually increased from 31 to 118 mg/g when the Cu concentration was increased from 1 to 20 mg/L. The aggregation and sedimentation of TiO_2 NPs were significantly increased after 48 h of co-exposure with the Cu at a concentration higher than 5 mg/L, as compared to the single TiO_2 NPs exposure. The increasing aggregation and sedimentation might reduce the bioavailability of TiO_2 NPs associated with the adsorbed Cu to soybeans, and consequently alleviate or even neutralize the enhanced toxicity and accumulation of Cu in soybeans exerted by the co-existing TiO_2 NPs. Our results thus suggest that consideration of the impact of TiO_2 NPs on the phytotoxicity of heavy metals, and specifically Cu, needs to be interpreted with care, and highlight the importance of integrating the interaction and fates of TiO_2 NPs and metals into their risk assessment.

1. Introduction

The rapid development of nanotechnology has led to an increasing amount of manufactured nanoparticles (NPs) being unintentionally discharged into the environment. Among the manufactured NPs, TiO_2 NPs undoubtedly are the most widely applied, with an estimation of 2.5 million tons to be produced annually by 2025 (Chavan et al., 2020; Robichaud et al., 2009). Given the large production of TiO_2 NPs, these NPs are inevitably released into the environment, with agricultural soil as a primary sink. It is estimated that there are 89 μg of TiO_2 NP every year to be accumulated in per kg of soil (Gottschalk et al., 2009). In the EU region, the total concentration of TiO_2 NPs in the soil can reach as high as 50 mg/kg (Meesters et al., 2016). Therefore, exposure to TiO_2 NPs is increasingly possible for crops. Several studies have demonstrated that TiO_2 NPs could be accumulated and translocated in edible plants even when the primary sizes of the particles were larger than the root apoplastic barriers (Larue et al., 2012; Kurepa et al., 2010). This raises a growing concern about the threat of TiO_2 NPs to food safety. Furthermore, due to their unique physicochemical characteristics, TiO_2 NPs have a high potential to interact with other co-existing pollutants, such as the so called "heavy metals" (Wang et al., 2019; Tan et al., 2017; Fan et al., 2016a). Subsequently, the bioavailability and toxicity of the co-existing metals to exposed organisms may be altered. For instance, some studies have reported that the co-exposure of NPs significantly

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https://doi.org/10.1016/j.ecoenv.2021.112312
Received 27 December 2020; Received in revised form 15 April 2021; Accepted 4 May 2021
Available online 11 May 2021

enhanced the accumulation and phytotoxicity of heavy metals via acting as metal carriers (Lian et al., 2020; Wang et al., 2018a). On the other hand, NPs have also shown to mitigate the uptake and toxicity of co-existing metals to plants due to absorb metals on NPs (Rossi et al., 2018; Ji et al., 2017; Wang et al., 2015). The contradicting effects indicate that more comprehensive studies involved in the joint effects of NPs and heavy metals on edible plants are needed to better elucidate the potential risk of the simultaneous presence of NPs and heavy metals to food safety.

Copper (Cu) is a naturally occurring metal and Cu pollution is common in the environment due to both geogenic and anthropogenic activities (Cao et al., 2018; Thounaojam et al., 2012). Recently, there have been some studies finding that TiO_2 NPs could modulate the adverse effects of excessive Cu to a series of aquatic organisms, such as *Daphnia magna* (Fan et al., 2011; Rosenfeldt et al., 2015a, 2016), *Mytilus galloprovincialis* (Torre et al., 2015) and *Gammarus fossarum* (Rosenfeldt et al., 2015b). However, the current knowledge in terms of the joint effects of TiO_2 NPs and Cu to edible terrestrial plants is rather limited. To the best of our knowledge, there is only one study investigating the combined effects of TiO_2 NPs and Cu on edible plant, in which a mitigation of toxicity of Cu to rice by the co-exposure of TiO_2 NPs was reported (Wang et al., 2015).

In addition, mechanistic insights into the interactions between TiO_2 NPs and Cu are lacking. Adsorption of heavy metals on NPs has been proposed as a key interaction to regulate the bioavailability and subsequent toxicity of heavy metals in organisms (Sebastian et al., 2019; Sharifan et al., 2018; Ji et al., 2017; Wang et al., 2015). On the one hand, heavy metals have a strong propensity to adsorb on co-existing NPs (Rossi et al., 2017; Fan et al., 2016b). On the other hand, adsorbed metals have the potential to alter the physicochemical characteristics of NPs (e.g., hydrodynamic diameter and zeta-potential) (Sharifan et al., 2018; Wang et al., 2018b). Consequently, the fate and bioavailability of the NPs together with the adsorbed metals may be altered. However, there is a lack of studies integrating the reciprocal effects of NPs and heavy metals on their fates in the environment, whilst specifically considering the joint toxicity of NPs and heavy metals to crops. This undoubtedly hinders to uncover the possible mechanisms behind the toxicological interactions of NPs and heavy metals in the environment.

In view of that, one of the objective of this study was to explore the particular impact of the co-presence of TiO_2 NPs and Cu ions on the toxic effects and accumulation of this heavy metal in soybeans. Soybean was selected as it is the fifth most produced crop in the world and accounts for 30% of the global production of vegetable oil (Rossi et al., 2018). Moreover, the reciprocal effects of TiO_2 NPs and Cu on their fates were monitored in a hydroponic system, including assessment of the adsorption of Cu onto TiO_2 NPs, TiO_2 NPs characterization, and quantification of the residual amounts of TiO_2 NPs and Cu in the exposure suspensions within the incubation period, with an attempt to better understand the joint toxicity and accumulation of TiO_2 NPs and Cu in soybean seedlings.

2. Materials and methods

2.1. Growth analysis of soybean plants

Soybean seeds (*Glycine max* L. cv. Nandou 12) were provided by Sichuan Engineering Research Center for Crop Strip Intercropping System (China). The seeds were disinfected with 0.5% (w/v) sodium hypochlorite solution for 20 min and then rinsed thoroughly with distilled water three times. The sterilized seeds were germinated on moistened filter paper in Petri dishes. The Petri dishes were covered with tinfoil for light blocking and cultured in a growth chamber at 25 ℃ for 5 d. The germinated seedlings with similar growth status were transplanted into 50 mL glass beakers filled with 10% strength Hoagland solution (pH 6.8) (Hoagland and Arnon, 1950). The TiO_2 NPs (anatase, purity $\geq$ 99%) were purchased from XFNANO Materials Technology Co., Ltd (Nanjing, China) with advertised particle size and surface area equaling 20–40 nm and 77.37 m^2/g, respectively. $CuSO_4.5H_2O$ was provided by the Sinopharm Chemical Reagent Co., China. Before transplanting the seedlings, the TiO_2 NP and Cu stocks were introduced into the 10% strength Hoagland solutions to achieve the target exposure concentrations, as follows: individually 10 mg/L TiO_2 NPs and Cu at 1, 2, 5, and 20 mg/L; combinations of 10 mg/L TiO_2 NPs + 1 mg Cu/L, 10 mg/L TiO_2 NPs + 2 mg Cu/L, 10 mg/L TiO_2 NPs + 5 mg Cu/L and 10 mg/L TiO_2 NPs + 20 mg Cu/L. Soybean seedlings were transplanted into the 10% strength Hoagland solutions without the addition of TiO_2 NPs and Cu were regarded as the control samples in the experiment. The concentration of 10 mg/L of TiO_2 NPs as selected in this study did not lead to growth differences of soybean seedlings, as compared to the control treatment. There were two seedlings planted in each beaker filled with 25 mL of the amended Hoagland solution. All the seedlings were cultured in the growth chamber at 25 °C under a 6000 lux light intensity for 6 d (16: 8 h light/dark photoperiod). The beakers were sealed with perforated parafilm to avoid high evaporation while maintaining air circulation. The culture media were refreshed every 2 days. After harvesting, the roots and shoots were rinsed with distilled water three times and their lengths were measured with a ruler. After being dried for 48 h at 70 °C, the dry weights of roots and shoots were determined.

2.2. Accumulation of Cu and Ti in soybean seedlings

After determining the dry weight of each seedling as described above, the two seedlings in each beaker were mixed to analyze the Cu and Ti contents associated with roots and shoots. The dry roots and shoots were digested at room temperature with 4 mL of 69% (v/v) HNO_3 and 2 mL of 30% (w/v) H_2O_2 overnight, and then the digests were heated at 180 °C on an electric hot plate until the digests turned achromatic or pale yellow. After cooling down to room temperature and dilution of the digests with 1% HNO_3 to 15 mL, the Cu and Ti contents in the digestion solution were quantified by inductively coupled plasma optical emission spectrometry (ICP-OES, iCAP 6300 DUO, Thermo, U.S. A.). To evaluate the reliability of the digestion method, four replicates of pure TiO_2 NPs were digested in the same method as mentioned above and determined by ICP-OES. Consistent results with the concentration of pure TiO_2 NPs were obtained.

To clarify in this study, the measured Cu and Ti contents associated with the soybean roots were defined as the sum of the metals internalized into the roots and adsorbed on the surface of roots. After rising the roots with distilled water, the TiO_2 NPs associated with adsorbed Cu might not be completely removed from the surface of roots. It has been reported that the surface adsorbed NPs can still exert adverse effects on the growth of plants by inhibiting the transportation of water and nutrients (Margenot et al., 2018; Servin and White, 2016; Dietz and Herth, 2011; Asli and Neumann, 2009). Therefore, both the internalized and adsorbed fractions of NPs were considered as being accumulated by the soybean roots in this study.

2.3. Characterization of TiO_2 NPs

Stock solutions of TiO_2 NPs (1000 mg/L) were freshly prepared in MilliQ water after 30 min of sonication in a water bath sonicator. Stock solutions of TiO_2 NPs and Cu were added into the 10% strength Hoagland medium in glass bottles to prepare the target exposure concentrations used for the growth analysis of soybeans, as depicted above. The particle size and shape of TiO_2 NPs in the 10% strength Hoagland medium were characterized using transmission electron microscopy (TEM, JEOL 1010, JEOL Ltd, Japan). The prepared TiO_2 NP suspensions were maintained in the growth chamber at 25 ℃ under a 6000 lux light intensity for 48 h (16 h light-8 h dark photoperiod). Within the 48 h of incubation, the hydrodynamic diameters of the TiO_2 NPs were determined in triplicate in the prepared culture medium at 1, 12, 24, and 48 h, respectively, by dynamic light scattering (DLS) analysis (Malvern, Instruments ltd., UK). At the same time points, the zeta-potential of each TiO_2 NP suspension was measured by the same instrument via electrophoretic light scattering. At each sampling timepoint (namely 1, 12, 24, and 48 h after preparation), a 5 mL sample of the suspension was collected carefully from the position approximately 2 cm below the surface of each suspension, and the sample was then digested by concentrated nitric acid for at least 1 d, before being subjected to ICP-OES analysis. Standard TiO_2 NPs solution was used to validate the digestion method. In this way, the contents of Ti remaining in the water column with varying concentrations of Cu during the 48 h incubation were monitored.

2.4. Adsorption of Cu on TiO_2 NPs

At the sampling time point of 48 h, after drawing the 5 mL of suspension used to analyze the residual TiO_2 NPs in each suspension as depicted above, a 10 mL sample was drawn from the water column of each TiO_2 NP suspension. The samples were then centrifuged at 16,089 *g* for 30 min, followed by filtering through a syringe filter with a 0.02 μm pore diameter (Anotop 25, Whatman, UK). Cu contents in the filtrates were determined by means of ICP-OES. The Cu concentrations determined in the filtrates were considered as being non-adsorbed. The amount of adsorption of Cu on TiO_2 NPs was determined by analysis of the mass difference between the initial Cu content and the non-adsorbed Cu content. It has been demonstrated that the adsorption of heavy metals on TiO_2 NPs could be fitted well by the Freundlich model (Chen et al., 2012). The adsorption isotherm of Cu on TiO_2 NPs was therefore fitted with the Freundlich equation:

$$q_e = KC_e^{1/n}$$

In this equation, q_e and C_e are the adsorbed and non-adsorbed amounts of Cu (mg/L) after 48 h of equilibration, respectively. K indicates the adsorption coefficient (mg/g) $(L/g)^n$, and 1/n refers to the Freundlich coefficient.

2.5. Statistical analysis

Statistical analysis was conducted with the SPSS 16.0 package for Windows. Two-way analysis of variance (ANOVA) was used to analyze the significance of the interaction effects between TiO_2 NPs and Cu on the growth characters of soybean plants, the Ti and Cu contents in soybean seedlings, as well as the translocation factors of Ti and Cu in soybean seedlings. One-way ANOVA and the *t*-test were applied to analyze the significance of the differences of the growth characters of soybean seedlings, the contents of Ti or Cu in soybean seedlings, the adsorption of Cu onto TiO_2 NPs, the hydrodynamic-diameters of TiO_2 NPs, and the residual of TiO_2 NPs remaining in the water column among different treatments. The significance level in all analysis was set at $\alpha = 0.05$. Except for the data of the amount of Cu adsorbed onto TiO_2 NPs after 48 h of equilibration, all data were expressed as mean values ± standard deviation (SD).

3. Results

3.1. Adsorption of Cu on TiO_2 NPs

The adsorption isotherm of Cu onto TiO_2 NPs is shown in Fig. 1. The amounts of adsorbed Cu onto TiO_2 NPs were gradually increased with an increase of the initial Cu concentration applied in the solutions (Fig. 1). Specifically, the amounts of adsorbed Cu onto TiO_2 NPs were around 31, 47, 79, and 118 mg/g at dissolved Cu concentrations of 1, 2, 5, and 20 mg/L, respectively, after 48 h of equilibration. However, the percentage of non-adsorbed Cu relative to the initial amount of Cu applied for exposure increased with increasing initial Cu concentrations (Table S1). In particular, there was around 67%, 76%, 84%, and 94% of

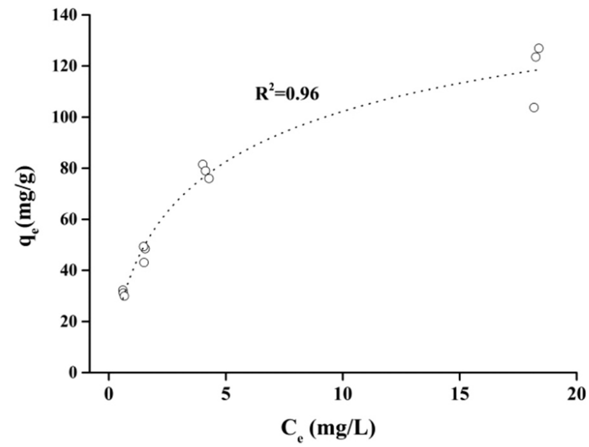

Fig. 1. The adsorption isotherm of Cu onto TiO_2 NPs. q_e (mg/g) is the amount of Cu adsorbed per unit weight of TiO_2 NPs after 48 h of equilibration, and C_e (mg/L) is the non-adsorbate Cu concentration in solution at 48 h.

Cu remaining in the supernatants after 48 h of incubation with the co-existing TiO_2 NPs when Cu was applied at concentrations of 1, 2, 5, and 20 mg/L, respectively.

3.2. Characterization of TiO_2 NPs

A TEM image of TiO_2 NPs in the 10% strength Hoagland solution after 1 h of incubation is presented in the Supplementary information (Fig. S1). The shape and primary size of the TiO_2 NPs were spherical and around 23 nm, respectively. As the incubation medium of the soybean seedlings was refreshed every 48 h, the aggregation status of TiO_2 NPs was monitored for 48 h. The DLS results showed that the hydrodynamic diameter of TiO_2 NPs without the addition of Cu shifted from 374 nm after 1 h of incubation to around 1064 nm after 48 h of incubation in the culture medium (Table 1). In the presence of 5 and 20 mg Cu/L, the hydrodynamic diameters of TiO_2 NPs increased to about 2000 nm after 48 h of incubation, and they were significantly higher than the hydrodynamic diameter in case of no addition of Cu to the suspension ($P < 0.05$) (Table 1). Additionally, the absolute value of the zeta-potential of TiO_2 NPs was reduced from 10 in the absence of Cu to 4 in the presence of 20 mg Cu/L after 48 h of incubation (Table 1). The absolute zeta-potential values of TiO_2 NPs when Cu was added at concentrations of 5 and 20 mg/L were significantly lower than the value without the addition of Cu ($P < 0.05$) (Table 1).

3.3. Growth analysis of soybean plants

The effects of Cu on the growth characteristics of soybean plants in the presence and absence of TiO_2 NPs are presented in Fig. 2. Upon the co-exposure of TiO_2 NPs and Cu, the changes of the root length and shoot height were significantly contributed by Cu, TiO_2 NPs and their interactions ($P < 0.05$) (Table 2). In the absence of TiO_2 NPs, the root length and shoot height were gradually decreased with an increase of the Cu concentration, with reductions of the root length and shoot height from 8.1 and 16.1 cm, at 0 mg Cu/L, to 5.3 and 7.7 cm at 20 mg Cu/L, respectively, after 6 days of incubation (Fig. 2A). Although the joint toxicity of TiO_2 NPs and Cu was mainly attributed to the impact of the heavy metal Cu (Table 2), the co-presence of TiO_2 NPs modulated the phytotoxicity of Cu. The specific effects of TiO_2 NPs on the phytotoxicity of Cu were dependent on the amount of Cu. When Cu was at levels of 1 and 2 mg/L, the presence of TiO_2 NPs significantly aggravated the phytotoxicity of Cu ($P < 0.05$), as reflected by the 15% and 12% more reduction of the soybean root lengths and by the 27% and 12% more decrease of the shoot heights as compared to the exposure to Cu alone at 1 and 2 mg/L, respectively (Fig. 2A and B). Additionally, in the

Table 1
Hydrodynamic diameter and zeta-potential of TiO_2 NPs upon varying Cu concentrations.

Treatment	Hydrodynamic diameter (nm)[a]			Zeta-potential (mV)[a]		
	1 h	24 h	48 h	1 h	24 h	48 h
TiO_2 NPs	374 ± 126	898 ± 105	1064 ± 206	−15 ± 1	−12 ± 2	−10 ± 1
TiO_2 NPs+ 1 mg Cu/L	410 ± 60	915 ± 84	1175 ± 274	−14 ± 1	−11 ± 1	−9 ± 1
TiO_2 NPs+ 2 mg Cu/L	391 ± 82	1203 ± 249	1459 ± 186	-14 ± 1	−10 ± 1	−8 ± 2
TiO_2 NPs+ 5 mg Cu/L	627 ± 103 *	1473 ± 151 *	1963 ± 388 *	−9 ± 2 *	−6 ± 2 *	−5 ± 1 *
TiO_2 NPs+ 20 mg Cu/L	848 ± 111 *	1850 ± 247 *	2275 ± 333 *	−11 ± 1 *	−6 ± 1 *	−4 ± 1 *

[a]Hydrodynamic diameter and zeta-potential are expressed as mean ± SD ($n = 3$). (*) Statistical differences of hydrodynamic diameter and zeta-potential were determined by one-way ANOVA, as compared to the treatment without the addition of Cu at the same incubation time ($P < 0.05$).

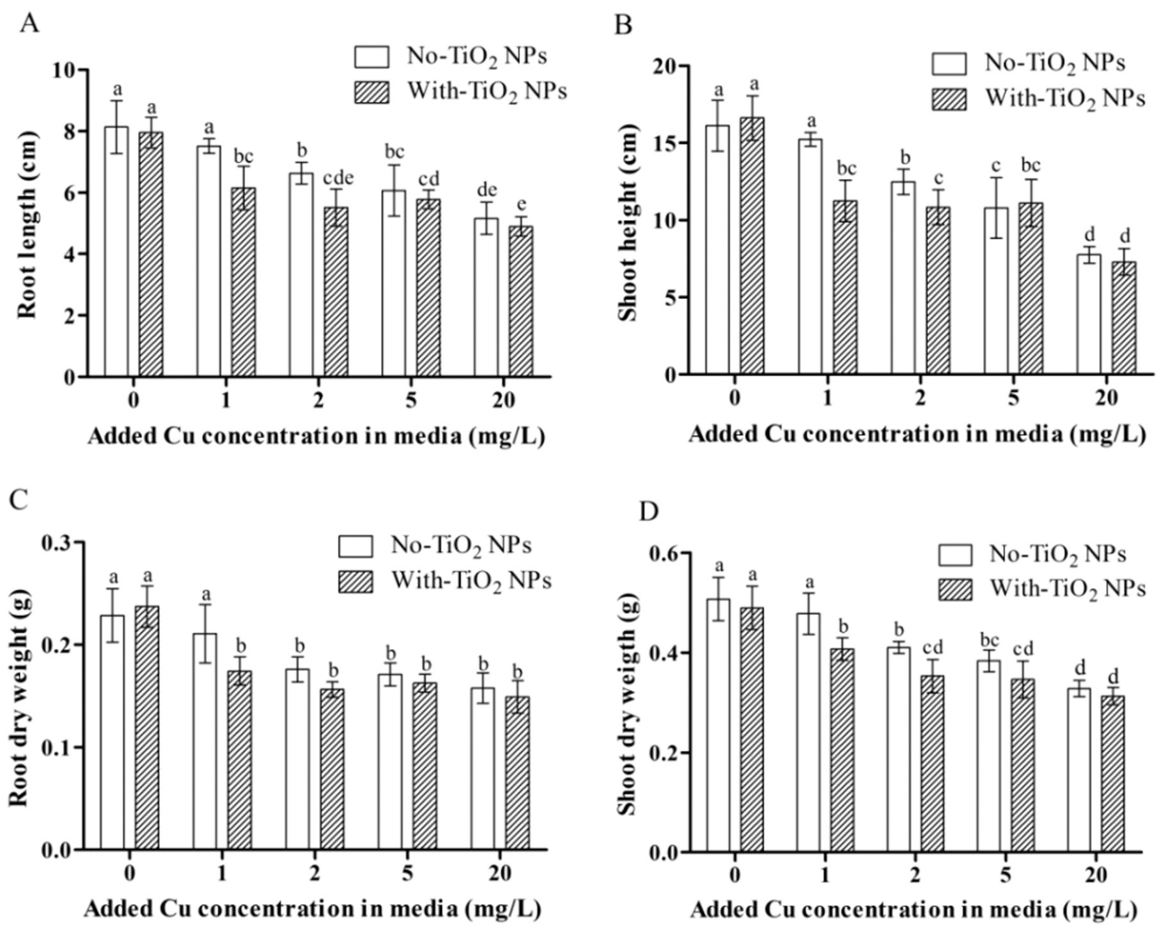

Fig. 2. Root length (A) and shoot height (B) of soybean plants after exposure to different concentrations of Cu and TiO_2 NPs. (C) and (D) represent the dry weights of roots and shoots determined after incubation, respectively. Data are presented as mean ± SD ($n = 6$). Different letters indicate significant differences among different treatments tested by one-way ANOVA and *t*-test ($P < 0.05$).

co-presence of TiO_2 NPs, the shoot biomass (dry weight) was significantly reduced when Cu was at concentrations of 1 and 2 mg/L ($P < 0.05$), compared to the shoot biomass of soybeans upon the treatments with Cu exposure alone. Similarly, the co-presence of TiO_2 NPs and Cu at a concentration of 1 mg/L significantly reduced the root biomass (dry weight) ($P < 0.05$), relative to the root biomass with Cu exposure alone (Fig. 2C and D). However, when Cu was present at concentrations of 5 and 20 mg/L, there were no significant differences in growth (including the lengths and dry weights of roots and shoots) between the treatments upon the co-presence of TiO_2 NPs and upon the Cu exposure alone ($P > 0.05$) (Fig. 2).

3.4. Uptake of Cu and Ti in soybean plants

Both the Cu and the Ti contents were determined in soybean roots and shoots (Fig. 3). The two-way ANOVA results showed that the Cu content in the soybean roots was only significantly affected by the Cu concentration upon the co-exposure of TiO_2 NPs and Cu ($P < 0.05$), while both the Cu concentration and the co-presence of TiO_2 NPs significantly affected the Cu content in the shoots ($P < 0.05$) (Table 2). The Cu content in the roots was gradually enhanced upon increasing Cu concentration in the exposure medium (Fig. 3A). When Cu was at a concentration of 2 mg/L in the exposure medium, the Cu content in roots was significantly increased from 77.8 mg Cu/kg without the addition of TiO_2 NPs to 113.1 mg Cu/kg upon the addition of TiO_2 NPs ($P < 0.05$) (Fig. 3A). However, there were no significant differences of Cu contents in roots between the treatments with and without the addition of TiO_2 NPs at other Cu concentrations employed in this study ($P > 0.05$) (Fig. 3A). In addition, when the Cu concentrations were 1 and 2 mg/L, the Cu contents in the shoots were significantly decreased from

Table 2
Significance (*F*-values) of the impact of TiO_2 NPs, Cu concentration and their interactions on measured variables based on two-way ANOVA.

Variables	Cu	TiO_2 NPs	Cu $\times TiO_2$ NPs
Root length	48.08**	19.59**	2.91*
Shoot height	78.85**	10.39**	6.40**
Root dry weight	20.19**	4.20 ns	1.39 ns
Shoot dry weight	30.15**	12.29**	0.93 ns
Root Cu content	294.52**	1.00 ns	1.93 ns
Shoot Cu content	83.06**	17.66**	2.17 ns
Root Ti content	19.66**	264.44**	20.74**
Shoot Ti content	15.77**	218.61**	16.72**
TF for Cu	87.21**	26.55**	8.26**
TF for Ti	0.29 ns	76.56**	0.66 ns

Significance levels: *P < 0.05; * *P < 0.01; ns, non-significant effect; TF = Translocation factor.

18.6 and 29.5 mg Cu/kg without the addition of TiO_2 NPs to 11.2 and 15.0 mg Cu/kg with the addition of TiO_2 NPs, respectively ($P < 0.05$) (Fig. 3B). The translocation factor (TF) is defined as the ratio of the metal content in shoots to the metal content in roots. Upon the co-exposure scenario, the TF of Cu in soybean plants was significantly affected by the factors of Cu concentration, TiO_2 NPs, and their interactions ($P < 0.05$) (Table 2). When Cu in the solutions was at levels of 1 and 2 mg/L, the TF was significantly decreased from 40.5% and 37.9% without the addition of TiO_2 NPs to 19.3% and 13.3% upon co-exposure to TiO_2 NPs, respectively ($P < 0.05$) (Fig. 4A).

The Ti contents in the soybean roots and shoots were significantly affected by the Cu concentration, the co-presence of TiO_2 NPs, and by their interactions ($P < 0.05$) (Table 2). When Cu was present at concentrations of 1 and 2 mg/L in the solutions, the Ti contents in the roots and shoots were significantly higher than those in case of addition of 0, 5, and 20 mg Cu/L in the solutions ($P < 0.05$) (Fig. 3C and D). Furthermore, the factor of Cu concentration ranging from 1 to 20 mg/L did not significantly affect the TF for Ti in the soybean plants ($P > 0.05$) (Fig. 4B).

3.5. Residual TiO_2 NPs in suspensions

The amount of TiO_2 NPs remaining in suspension decreased over time (Fig. 5). No matter whether Cu was co-present in the suspension, the sedimentation of TiO_2 NPs occurred mainly within the first 12 h of incubation (Fig. 5). Furthermore, it is worth to note that after 48 h of co-exposure the amount of residual TiO_2 NPs in suspension tended to be reduced with an increasing content of Cu applied in the solution (Fig. 5). As specific values, at levels of 5 and 20 mg Cu/L, there were around 28% and 22%, respectively, of the initially added amount of TiO_2 NPs remaining in the suspensions after 48 h of incubation. These levels were significantly lower than the residual amount of Ti without the addition of Cu to the suspension (around 41% still present in suspension after 48 h of equilibrium, $P < 0.05$) (Fig. 5).

4. Discussion

Even though in the current study the TiO_2 NPs at a concentration of 10 mg/L did not show significantly adverse effects on the growth indices (plant height and dry weight) of soybean seedlings, it was found that the TiO_2 NPs could aggravate the toxic effects of the co-existing heavy metal Cu on the growth of the soybean seedlings (Fig. 2). Especially when Cu was present at concentrations of 1 and 2 mg/L, the co-presence of TiO_2 NPs significantly aggravated the phytotoxicity of Cu, relative to the

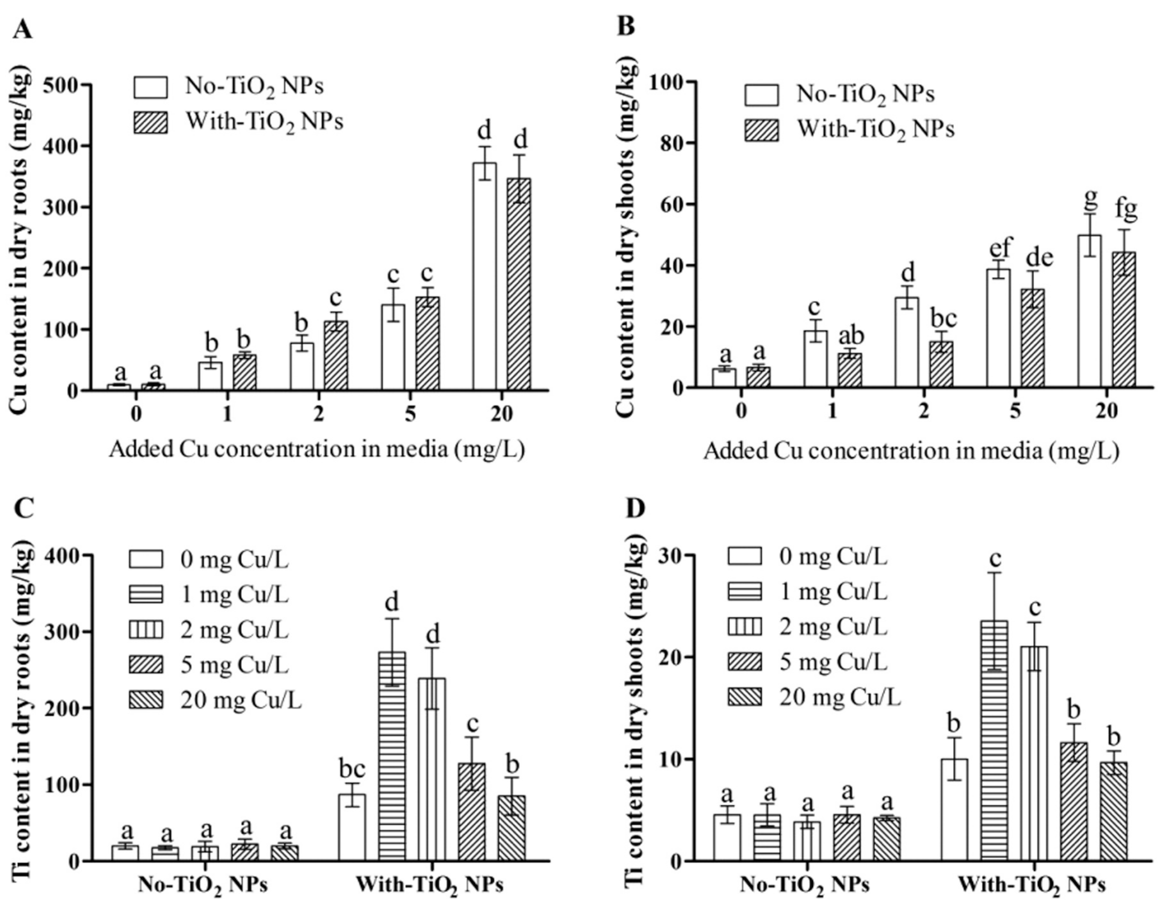

Fig. 3. The Cu and Ti contents in roots and shoots of soybean seedlings in the treatments with or without the addition of 10 mg/L of TiO_2 NPs. Data are expressed as mean ± SD (*n* = 3). Different letters indicate significant differences among different treatments tested by one-way ANOVA and *t*-test ($P < 0.05$).

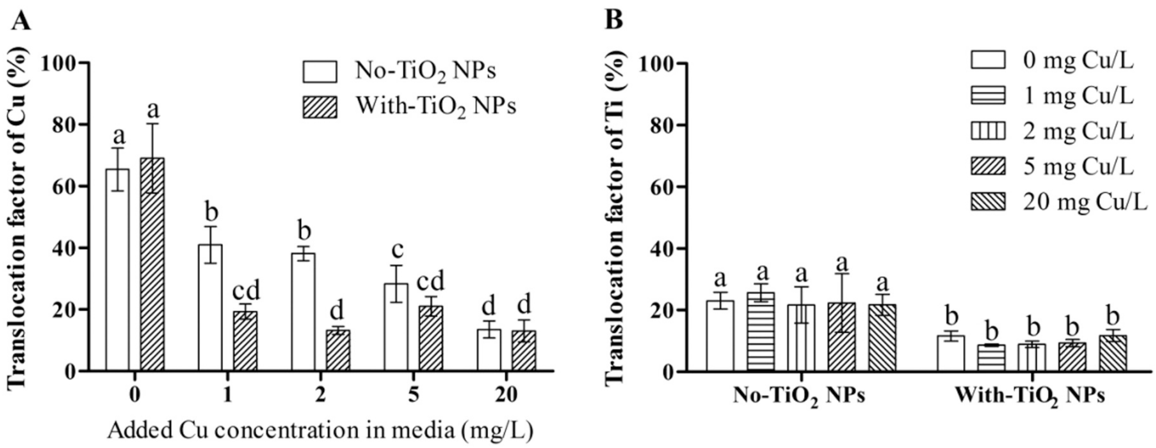

Fig. 4. Translocation factors of Cu (A) and Ti (B) in the soybean seedlings. Data are expressed as mean ± SD ($n = 3$). Different letters indicate significant differences among different treatments tested by one-way ANOVA and *t*-test ($P < 0.05$).

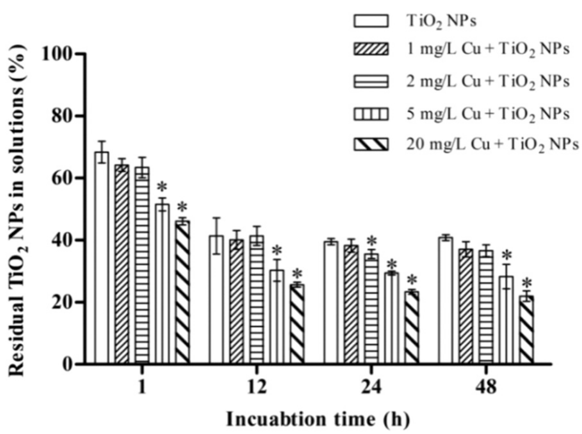

Fig. 5. Percentages of suspended TiO_2 NPs in the culture medium in the presence of varying amounts of Cu within the 48 h of incubation, relative to the initial TiO_2 NPs concentration (10 mg/L). The data are expressed as mean ± SD ($n = 3$). The black asterisks indicate a statistically different percentage of residual TiO_2 NPs in suspension ($P < 0.05$) as compared to the control (the treatment without the addition of Cu).

phytotoxicity induced by Cu alone ($P < 0.05$) (Fig. 2). Similarly, Lian et al. (2020) found that TiO_2 NPs (100 mg/L) significantly aggravated the toxicity of Cd to maize, with a much lower plant biomass in the co-exposure scenario than in the scenario with Cd exposure alone. The authors attributed the synergistic toxic effects of TiO_2 NPs and Cd to reciprocally promoting accumulation of Ti and Cd in maize plants, which subsequently induced a higher oxidative stress and synergistically aggravated the loss of plasma integrity. Fan et al. (2011) also found that TiO_2 NPs at a considerably safe concentration remarkably enhanced the toxicity of Cu to organisms (*Daphnia magna*). The authors attributed the aggravation of toxicity in the co-exposure of TiO_2 NPs and Cu both to the improved bioaccumulation of Cu in daphnids, and to the competition of TiO2 NP with Cu to bind with metallothionein, which is a cysteine-rich protein and plays an important role in detoxifying excessive metals in organisms. In our study, it was also observed that the co-exposure of TiO_2 NPs and Cu at levels of 1 and 2 mg/L significantly promoted the contents of Ti and Cu in roots, as compared to the individual TiO_2 NPs or Cu exposure (Fig. 3A and C). However, the reduced accumulation of metal ions in the co-exposure of NPs in plants due to the formation of NP-metal complexes has also been widely reported by some other studies (Sharifan et al., 2020; Katiyar et al., 2020; Cai et al., 2017). Although the precise mechanisms underlying the penetration of NPs through root apoplastic barriers (including cell walls, suberin, cutin, and casparian strip) remain unclear, an increasing number of studies have found that NPs with a primary size larger than the size exclusion limit (SELs) of plant root apoplastic barriers can still be internalized into cytoplasm (Lian et al., 2020; Ma and Yan, 2018; Larue et al., 2012). Some possible mechanisms underlying the mutually promoted accumulation of TiO_2 NPs and Cu in plants have been suggested. Specifically, a first aspect to be taken into account is that electrons and electron holes can be generated on the surface of TiO_2 NPs, which can induce the production of reactive oxygen species (ROS) (Fenoglio et al., 2009; Guo et al., 2006). Added to that, it has to be considered that electrons and electron holes generated on the surface of TiO_2 NPs can recombine to recover the original constitution of TiO_2 NPs (Fan et al., 2016a). However, the Cu ions adsorbed onto the TiO_2 NPs surface can scavenge the electrons released by TiO_2 and thus reduce the chance of recombination (Litter, 1999), leading to more electron holes formed. Consequently, there would be increasing ROS generated when Cu ions and TiO_2 NPs are co-existing (Chen et al., 1997). ROS have the potential to loosen plant cell walls by unspecific cleavage of polysaccharides (Fry, 1998), which may allow TiO_2 NPs and Cu to penetrate into roots more easily, and further induce a higher accumulation of Ti and Cu in plants. In our study, the adsorption of Cu ions onto TiO_2 NPs was monitored, finding that the percentage of Cu adsorbed onto TiO_2 NPs, relative to the initial Cu concentration applied in solutions, was decreased with increasing initial concentrations of Cu (Table S1), while the contents of Cu adsorbed onto TiO_2 NPs were gradually increased with increasing initial Cu concentrations (Fig. 1). Accordingly, the increasing adsorption of Cu on TiO_2 NPs may result in the phenomenon of promoting the penetration of Ti together with the adsorbed Cu into the roots when Cu was at levels of 1 and 2 mg/L (Fig. 3A and C). Also relevant, the transportation efficiency from roots to shoots of the heavy metals adsorbed onto NPs would be reduced (Rossi et al., 2019, 2018; Cai et al., 2017). This is consistent with the results indicating that the co-exposure of TiO_2 NPs (when Cu was at levels of 1 and 2 mg/L) significantly reduced the translocation factor for Cu in soybean plants ($P < 0.05$) (Fig. 4A). However, our results were not fully in agreement with the interpretation that Cu ions adsorbed onto TiO_2 NPs would allow more Ti and Cu to penetrate in roots. In fact, when Cu was present at 5 and 20 mg/L, the co-presence of TiO_2 NPs did not consistently result in a significantly higher amount of Ti and Cu contents in the soybean roots, as compared to the individual exposure of TiO_2 NPs or Cu ($P > 0.05$) (Fig. 3A and C). This is in line with the finding that there was no statistically significant differences of phytotoxicity induced by Cu at 5 and 20 mg/L upon co-exposure with the TiO_2 NPs as compared to the treatments of Cu exposure alone ($P > 0.05$) (Fig. 2).

To further interpret the joint toxicity and accumulation of TiO_2 NPs and Cu in soybeans, the reciprocal effects of Cu and TiO_2 NPs on their fates were monitored. In this study, at a high concentration of Cu ($\geq$ 5 mg/L), the Cu adsorbed onto the TiO_2 NPs significantly enhanced the zeta-potential of the TiO_2 NP suspension, as compared to other treatments including the suspensions with the TiO_2 NPs alone and with the co-exposure of the NPs and Cu at 1 and 2 mg/L ($P < 0.05$) (Table 1). This resulted in reduction of the electrostatic repulsion between TiO_2 NPs and improvement of the aggregation of TiO_2 NPs. Consequently, the sedimentation of TiO_2 NPs at a relatively high concentration of coexisting Cu ($\geq$ 5 mg/L) was higher. Similarly, Wang et al. (2019) found that only when the co-existing Pb^{2+} was at a level higher than 2 mmol/L, the aggregation of TiO_2 NPs (800 μg/L) was evidently promoted. This indicates that only when the concentration of counter-ion exceeds a certain level can evidently promote the aggregation of NPs. In this study, a high level of Cu (5 and 20 mg/L) induced a higher extent of sedimentation of TiO_2 NPs is likely to have reduced the bioavailability of the particles together with the adsorbed Cu ions to organisms (Torre et al., 2015; Rosenfeldt et al., 2014). Reduction of the bioavailability of TiO_2 NPs together with the adsorbed Cu may consequently alleviate or even neutralize their promoting role with regard to penetration of the soybean roots upon co-exposure. This elucidation seemingly well accounts for the results of non-significant mutual effects of the TiO_2 NPs and Cu on their contents in soybean roots in the co-exposure of TiO_2 NPs and Cu at 5 and 20 mg/L ($P > 0.05$) (Fig. 3A and C). However, further evidence in terms of the physical-chemical interactions between TiO_2 NPs and co-existing heavy metals (Cu in the current study) is needed to deeply understand the underlying mechanisms of the specific impact of TiO_2 NPs on the phytotoxicity of such pollutants.

In summary, the co-presence of TiO_2 NPs resulted in the alterations of toxicity and contents of Cu ions in soybean plants. Such alterations were dependent on the initial concentration of Cu for co-exposure. At relatively lower concentrations of Cu (namely 1 and 2 mg Cu/L), the co-presence of TiO_2 NPs significantly enhanced the phytotoxicity of Cu ($P < 0.05$), while at 5 and 20 mg Cu/L, the significantly aggravated toxicity due to the co-presence of TiO_2 NPs disappeared ($P > 0.05$). The reciprocal effects of TiO_2 NPs and Cu on their fates were further monitored. The co-presence with TiO_2 NPs resulted in a gradual increase of adsorption of Cu ions onto TiO_2 NPs upon increasing Cu concentration. The adsorption of Cu onto TiO_2 NPs did not reduce the bioavailability of Cu and TiO_2 NPs for soybean roots. Instead, the contents of Ti together with the adsorbed Cu ions in soybean roots were significantly increased upon the co-exposure of TiO_2 NPs and Cu at 1 and 2 mg/L ($P < 0.05$). However, the increase of Ti and Cu concentrations in soybean roots did not occur when Cu was applied at concentrations of 5 and 20 mg/L. This may be caused by the fact that the larger amounts of Cu adsorbed onto TiO_2 NPs when the Cu concentration equaled 5 and 20 mg/L, showed a higher tendency to reduce the zeta-potential of the TiO_2 NPs and subsequently led to a higher extent of aggregation and sedimentation of TiO_2 NPs, as compared to the treatments with the co-exposure of TiO_2 NPs and Cu at concentrations of 1 and 2 mg Cu /L. This may consequently alleviate or even neutralize the promoting toxicity and accumulation of Cu in plants upon the co-exposure of TiO_2 NPs and Cu at a relatively low concentration. Our results thus suggest that the potential risk of the co-exposure of TiO_2 NPs and Cu to edible plants needs to be carefully considered. Future study is needed to confirm whether similar interactions between TiO_2 NPs and the co-occurrence of metals, and specifically Cu, in a soil-plant system can be observed.

CRediT authorship contribution Statement

Yinlong Xiao: Conceptualization, Methodology, Writing, Funding acquisition; **Ying Du**: Investigation, writing - original draft, Funding acquisition; **Yue Xiao**: Investigation; **Xiaohong Zhang**: Resources; **Jun Wu**: Formal analysis, Resources; **Gang Yang**: Analysis, Funding acquisition; **Yan He**: Investigation; **Yaoyu Zhou**: Writing - review & editing; **Willie J.G.M. Peijnenburg**: Writing - review & editing; **Ling Luo**: Supervision, conceptualization, resources.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work was funded by the National Natural Science Foundation of China (Grant No. 21906109) and by the Power Construction Corporation of China (Grant No. DJ-ZDXM-2019-42) as well as by the National College Students Innovation and Entrepreneurship Training Programs (Grant No. 201910626131).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2021.112312.

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